Investigating alanine variant impact in the ligand binding domain of the nuclear receptor FXR

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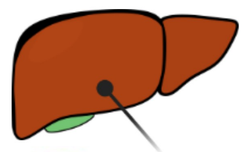
Introduction

The **farnesoid X receptor (FXR) is a vital nuclear receptor within the regulation of bile homeostasis** in the liver, balancing the necessity of bile acids for lipid digestion and the absorption of fat-soluble vitamins while high bile acid levels can have toxic effects due to their detergent nature. **Bile acids act as endogenous FXR agonists**, which leads to a downregulation of the synthesis of novel bile acids, increased canalicular export and reduced basolateral import of bile acids.

In the ligand binding domain (LBD) of FXR, a conformational change occurs from the **inactive to the active state, where helix 12 (H12) binds to the LBD core** and forms an interaction surface for the binding of coactivation peptides via a conserved motif. We set up an alanine mutational scan of the LBD using an experimental luciferase-based assay to investigate the position residue effect in detail. Variants with unexpected experimental results, contradicting literature indications, or with severe effects were further investigated in **unbiased Molecular Dynamics (MD)** simulations using AMBER23. Based on our previous work on the transition pathway from the inactive to the active state, we analysed the **variants T288A, K321A and H344A in both the inactive and active state** to explicitly model their potential impact on the activation pathway.



Bile acids for lipid digestion

Toxicity of bile acids

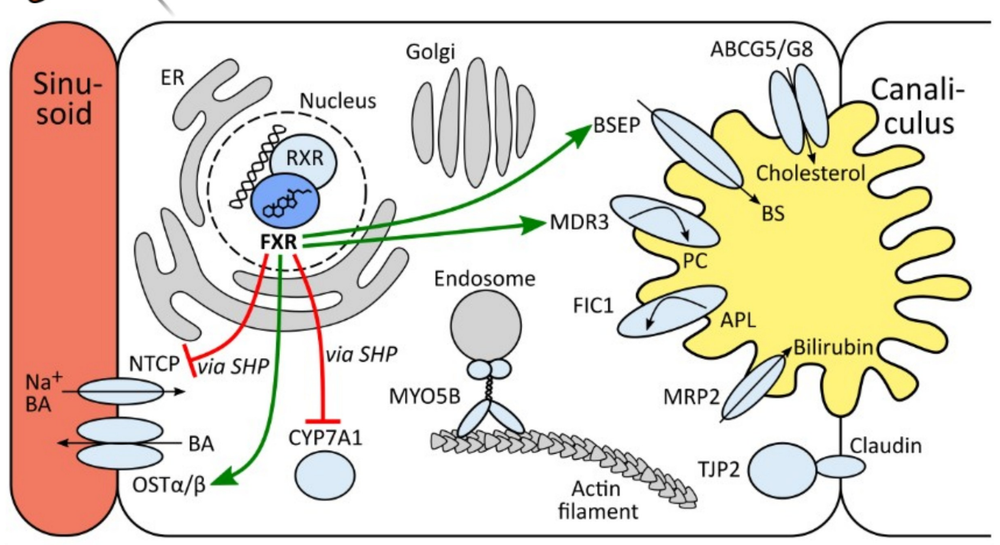


Fig. 1: Overview of relevant proteins within liver hepatocytes involved in bile homeostasis and the transcriptional influence of FXR on several key proteins like the bile salt export pump (BSEP), which transports bile salts (BS) into the canalicular lumen, the multidrug resistance protein 3 (MDR3), which flops phosphatidylcholine (PC) in the membrane, Cholesterol 7 alpha-hydroxylase (CYP7A1) as well as sodium taurocholate cotransporting polypeptide (NTCP) and organic solute transporters (OST) at the basolateral side. FXR acts mainly as heterodimer with retinoid X receptor (RXR) as a bile acid-receptive receptor to maintain the critical balance

Experimental setup

Transfection of HEK293 cells with FXR wildtype (WT) or variants and a plasmid containing FXR-target BSEP-promoter and Luciferase ⟶ Luc readout correlates with FXR activity.

Unbiased MD simulations of FXR WT and variants in inactive and active state using AMBER23, with a setup of 12 replica for 2µs each. To drive the systems to the active conformation, the agonist CDCA and a short coactivation peptide sequence is present. RESP-derived parameters for CDCA were obtained in previous work using the R.E.D. server with the 6-31G(d) level of theory for Gaussian16 and employed within the current project.

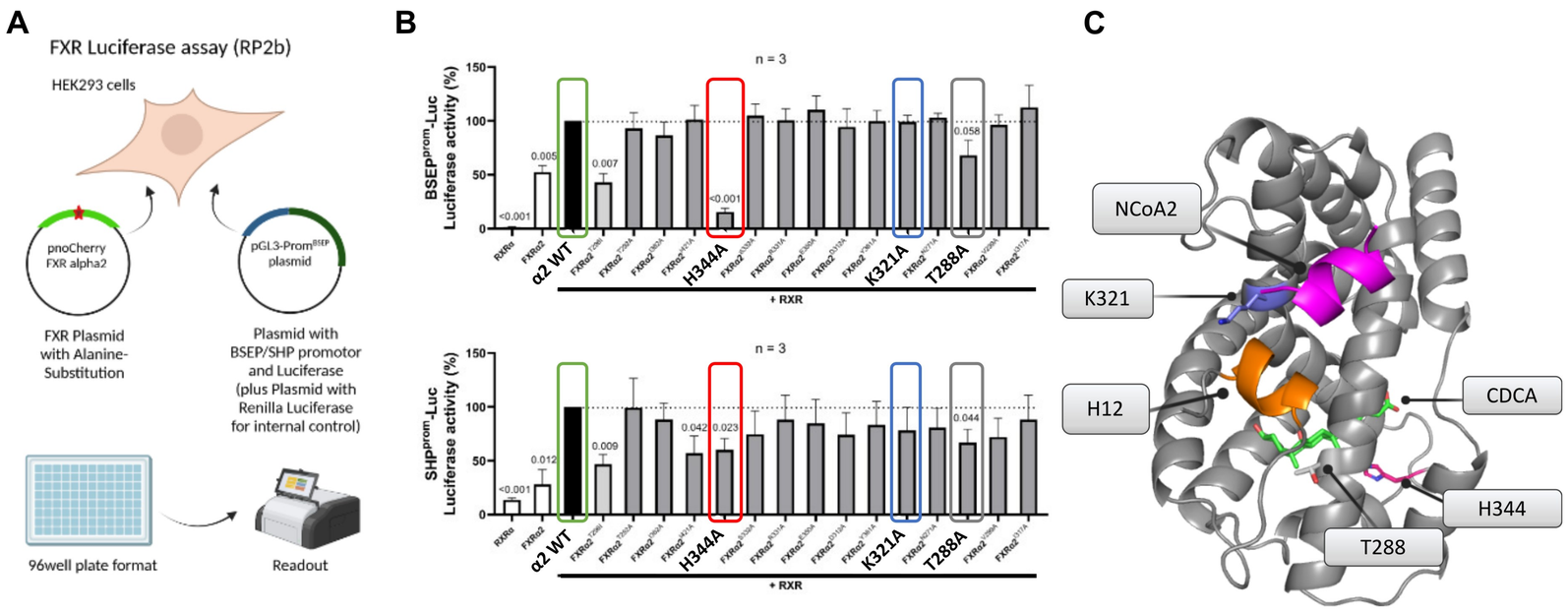


Fig. 2: Experimental assay data from first alanine variants within the FXR LBD. A) Overview of cellular luciferase-based assay to investigate FXR WT and variant protein transactivation activity. B) Measured protein transactivation activity of FXR LBD of WT (green box) and first alanine variants. Variants with unexpected and/or strong results are additionally highlighted, H344A (red box), K321A (blue box) and T288A (grey box). C) Localization of the variants H344A (red), K321A (blue) and T288A (grey) in the protein structure of FXR LBD in the active conformation. Additionally marked are the helix 12 (H12, orange) due to its special relevance for the active state of the protein, the short nuclear coactivation peptide 2 (NCoA2, magenta) and the endogenous agonist chenodeoxycholic acid (CDCA).

Results

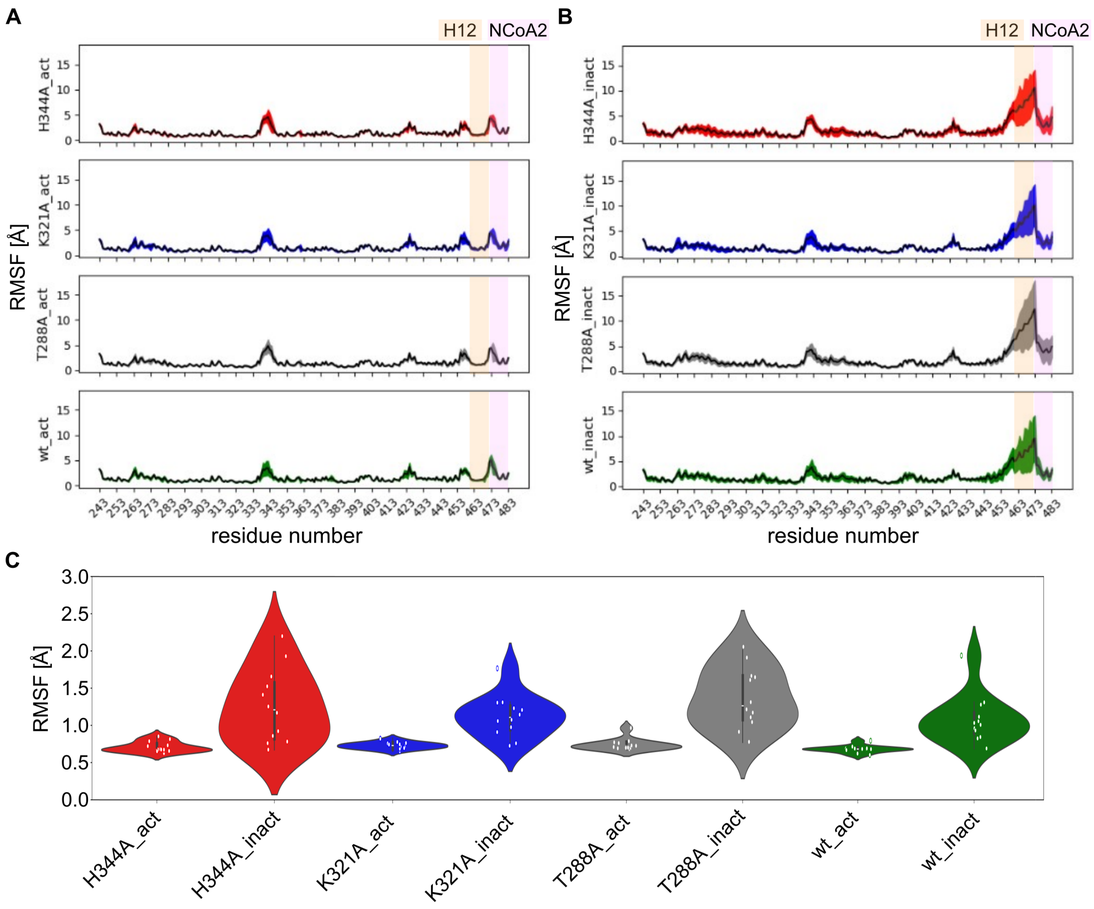


Fig. 3: Root mean squared fluctuation (RMSF) of the FXR LBD, the NCoA2 peptide and the CDCA ligand during MD simulations. A) Analysis for systems started in the active conformation (wt/variant_act naming). B) Analysis for systems started in the inactive conformation (wt/variant_inact naming). The helix 12 (H12, orange) and the coactivation peptide 2 (NCoA2) sequence (magenta) are highlighted. C) Analysis of the mobility of agonist CDCA during 12 MD replica with 1 µs simulation time each.

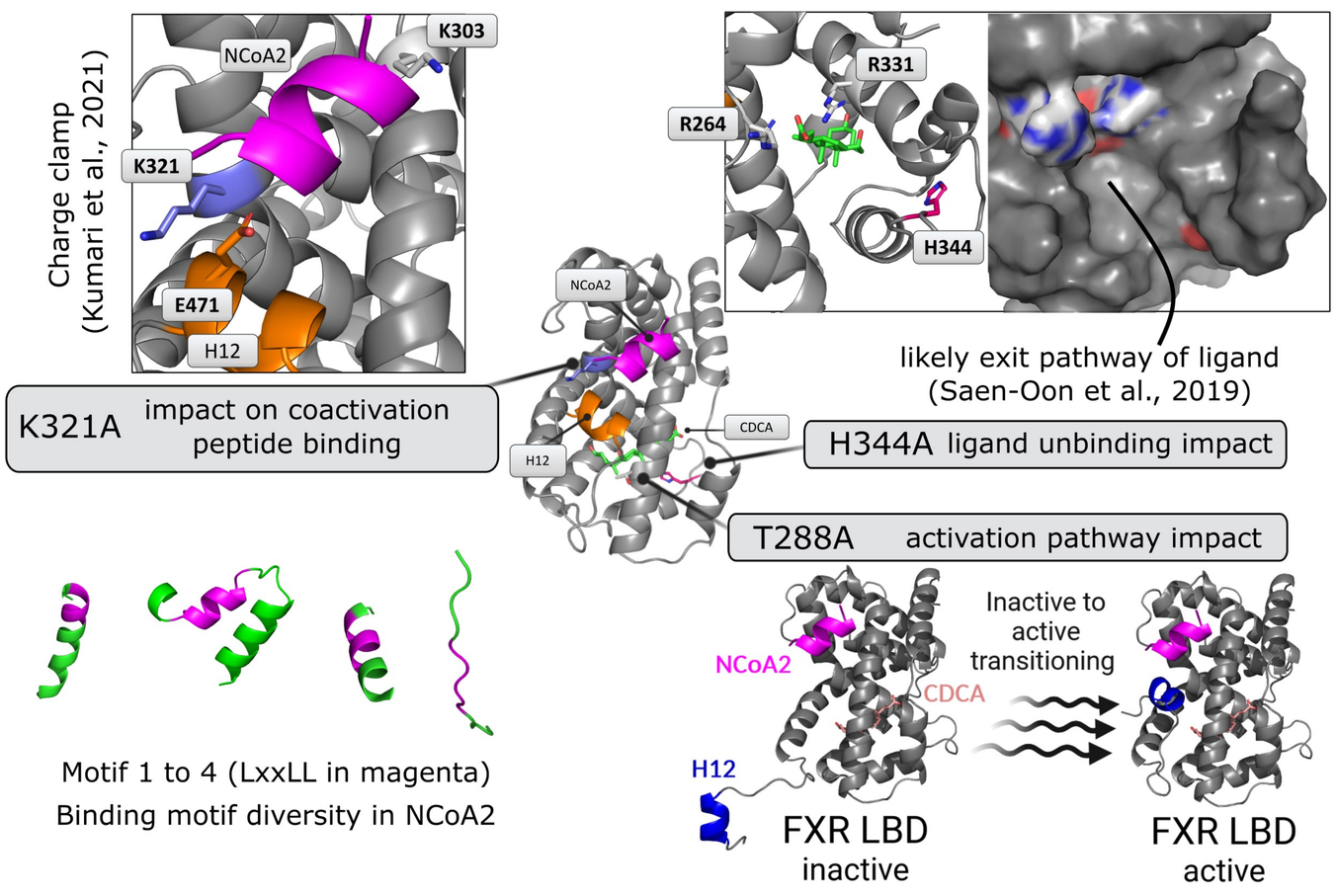


Fig. 4: Overview of the expected molecular mechanism for each variant. Variant K321A was expected to impact a critical charge clamp formation, described as important for coactivation peptide motif binding (via a conserved LxxLL motif) (Kumari et al., 2021). In our MD simulations and *in vitro* results however show no dysfunction, indicating that the length and/or sequence of the binding motif may impact results. Binding motifs within NCoA2 are highlighted in magenta and shown based on AlphaFold3 structure prediction in NCoA2/FXR dimer. Variant H344A might impact ligand unbinding based on previous MD studies in the NR RORγ (Saen-Oon et al., 2019). Variant T288A was expected to impact the transition from inactive to active state based on previous work on this critical transitioning (Behrendt et al., 2024).

Outlook

- Investigating differences between MD setups to evaluate K321A variant impact: using different coactivation peptide sequences to include motif diversity.
- Evaluating variant impact in detail – effect on charge clamp, transitioning pathway and ligand unbinding.
- Broadening the investigation to other variants of interest – like K303A.

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References & Funding

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